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Ultrastructural changes in the midguts of Hessian fly larvae feeding on resistant wheat

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ABSTRACT

The focus of the present study was to compare ultrastructure in the midguts of larvae of the Hessian fly, *Mayetiola destructor* (Say), under different feeding regimens. Larvae were either fed on Hessian fly-resistant or -susceptible wheat, and each group was compared to starved larvae. Within 3 h of larval Hessian fly feeding on resistant wheat, midgut microvilli were disrupted, and after 6 h, microvilli were absent. The disruption in microvilli in larvae feeding on resistant wheat were similar to those reported for midgut microvilli of European corn borer, *Ostrinia nubilasis* (Hubner), larvae fed a diet containing wheat germ agglutinin. Results from the present ultrastructural study, coupled with previous studies documenting expression of genes encoding lectin and lectin-like proteins is rapidly up-regulated in resistant wheat to larval Hessian fly, are indications that the midgut is a target of plant resistance compounds. In addition, the midgut of the larval Hessian fly is apparently unique among other dipterans in that no peritrophic membrane was observed. Ultrastructural changes in the midgut are discussed from the prospective of their potential affects on the gut physiology of Hessian fly larvae and the mechanism of antibiosis in the resistance of wheat to Hessian fly attack.

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1. Introduction

The Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae), is present in all of the wheat production areas within the United States and is the most important insect pest of wheat in the eastern soft red winter wheat region (Ratcliffe et al., 2000). Females oviposit on the leaves of wheat, and neonate larvae crawl down the leaves to enter the leaf sheath, where they feed near the crown or at nodes. All damage to wheat is due to larval feeding. In the fall, infestation results in increased permeability in the leaf sheath (Shukle et al., 1992; Subramanyam et al., 2007) as well as stunting and development of a dark green color in infested primary shoots or tillers (Byers and Gallun, 1972) and in spring, larval feeding prevents normal elongation of the culm and transport of nutrients to the developing kernel (Buntin, 1999). Adult Hessian flies are small dark midges approximately 3 mm long that do not feed and live for only 2–3 days after emergence.

Genetically resistant wheat is the most economical and environmentally sound method for control of Hessian fly particularly in the southeastern states where "fly-free" dates for delayed planting to escape infestation of the crop are not as effective as in more northern states (Ratcliffe and Hatchett, 1997; Flanders et al., 2000). To date, 33 resistance genes (*H1–H32* plus *Hdic*) have been identified for protection of wheat from Hessian fly attack (Sardesai et al., 2005; Liu et al., 2005). Resistance in the plant is expressed as larval antibiosis and is generally controlled by single genes that are partially to completely dominant (Gallun, 1977; Harris et al., 2003), while virulence in the insect is controlled by non-allelic recessive genes at single loci and operates on a gene-for-gene basis with respect to resistance (Hatchett and Gallun, 1970; Formusoh et al., 1996; Zantoko and Shukle, 1997). While genetic resistance is the most effective method of control, deployment of resistance has led to the selection of genotypes (biotypes) of the insect that can overcome formerly resistant wheat. This emergence of virulent genotypes is a threat to the future durability of resistance and wheat production (Martin-Sanchez et al., 2003).

The midgut and salivary glands are important interfaces between phytophagous insects and their host plants, with the midgut functioning in digestion of ingested food, absorption of nutrients, and detoxification of host allelochemicals (Terra and Ferreira, 1994; Barbehenn, 2001; Howe and Jander, 2008). Early studies of the larval Hessian fly described the general morphology of the alimentary tract including the midgut (Haseman, 1930) as

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well as the structure of the larval mouthparts (Hatchett et al., 1990) with the goal of revealing the feeding mechanism of larvae and any relationship to the susceptibility of different wheat cultivars to infestation. Recent studies of the midgut transcriptome have focused on characterization of genes involved in digestion, detoxification, antioxidant defense, and immune response and their expression during development and in larvae feeding on susceptible and resistant wheat (Mittapalli et al., 2005a,b, 2006, 2007a,b; Zhu et al., 2005; Liu et al., 2006; Chen et al., 2006). Such analyses have provided information on the physiological and biochemical processes undertaken in the Hessian fly larval midgut and can identify specific targets for genetically engineered resistance to complement native resistance in wheat.

Because of its role as an interface between the insect and its host plant, the midgut of the larval Hessian fly has been assumed to be an important target for host-plant allelochemicals in the incompatible interaction on resistant wheat. Previous studies have documented the expression of genes encoding lectin and lectin-like proteins is rapidly up-regulated in resistant wheat plants in response to attack by larval Hessian fly (Subramanyam et al., 2006; Giovanini et al., 2007), suggesting lectin and lectin-like proteins play a pivotal role in the defense response of resistant wheat. Further, disruption of midgut microvilli and purging of midgut contents have been reported in European corn borer, *Ostrinia nubilasis* (Hubner), and *Drosophila melanogaster* Meigen larvae fed a diet containing wheat germ agglutinin (WGA) (Harper et al., 1998; Li et al., 2009) and in the rice brown planthopper, *Nilaparvata lugens* (Stal), fed on a diet containing *Galanthus nivalis* agglutinin (GNA) (Powell et al., 1998). These data suggest the hypothesis the midgut of the larval Hessian fly is a target for toxic compounds expressed in resistant wheat is plausible; however, to date there are no data directly supporting this hypothesis. The objectives of the present study were to: (1) discover whether ultrastructural changes occurred in the midguts of Hessian fly larvae feeding on resistant wheat compared to larvae feeding on susceptible wheat and larvae subjected to starvation; (2) examine the temporal pattern for ultrastructural changes occurring in the midgut.

2. Materials and methods

2.1. Insect and plant material

Hessian fly Biotype L was used in the present study and was maintained in culture under greenhouse condition on wheat cultivar 'Magnum' (carrying resistance gene *H5*). Biotype L is defined as being able to survive on and stunt wheat carrying resistance genes *H3*, *H5*, *H6*, *H7H8* but is unable to survive (avirulent) on wheat carrying resistance gene *H9*. Two near isogenic wheat germplasm lines 'Newton' (carrying no genes for resistance) and 'Iris' (carrying resistance gene *H9*) (Patterson et al., 1994) were used to provide compatible and incompatible Hessian fly–wheat interactions, respectively. Biotype L on susceptible Newton wheat represented a compatible interaction, and Biotype L on resistant Iris wheat represented an incompatible interaction. To subject larvae to starvation, neonate larvae that had not entered the leaf sheath to establish a feeding site were collected and placed on moist filter paper in a 10 cm Petri dish.

2.2. Light microscopy

To document the overall morphology of the Hessian fly larva's alimentary tract and midgut the alimentary tracts plus salivary glands, Malpighian tubules, and hindgut were dissected from a late 1st-instar larva (5-days old) in a manner similar to that described by Grover et al., 1988. Briefly, dissection was conducted in cold Schneider's insect medium (Sigma–Aldrich, http://www.sigmaaldrich.com/sigma-aldrich/home.html) in a well slide by grasping the posterior end of a larva with a pair of watch-makers forceps and the anterior end with another pair of forceps. The alimentary canal, salivary glands, Malpighian tubules, and hindgut were then pulled from the anterior end along with the mouth parts and a small fragment of cuticle from the anterior end. The dissected alimentary tract was photographed utilizing an Olympus SZX12 stereoscopic microscope and camera system (Olympus, http://www.olympusamerica.com/seg_section/seg_stereo.asp).

2.3. Transmission electron microscopy

Larvae for transmission electron microscopy were collected from within the leaf sheath of susceptible Newton and resistant Iris wheat at 3, 6, and 24 h after egg hatch by dissecting open the leaf sheath with forceps, removing the exposed larvae with a small brush, and placing them on double sided tape attached to a glass microscope slide. Larvae subjected to starvation were collected by cutting off a mature leaf blade on which eggs had been laid and allowing the hatched larvae to crawl down the leaf into deionized water. Larvae were then removed from the water and placed on moist filter paper contained in a Petri dish. To prepare larvae for transmission electron microscopy they were removed from the moist filter paper and placed on double sided tape at the same times post-egg hatch as were larvae from Newton and Iris wheat.

The small size of early 1st-instar Hessian fly larvae (~500 μm in length and 50 μm in width) made dissection of the midgut difficult, and when removed from larvae, midguts frequently became distorted. To overcome this difficultly we severed the anterior tip of larvae immobilized on the double sided tape with a 26-gauge syringe needle and immediately applied a 3–5 μl drop of cold fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 01. M Na-cacodylate buffer containing 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.25% NaCl, pH 7.4) to cover the larva. The fixative released the pierced larva from the tape and it was transferred using the syringe needle to 500 μl of cold fixative contained in a 1.5 ml microfuge tube kept on ice. Generally, 20–30 larvae were collected per treatment (i.e. from susceptible Newton wheat, resistant Iris wheat, or starved) at each time post-egg hatch.

After collection, primary fixation of specimens was done by a microwave, vacuum method using a Pelco 3415 Research Microwave System (Ted Pella Inc., http://www.tedpella.com/). In brief, this consisted of two 40 s pulses at a power setting of one, followed by 3 min off after each pulse and a vacuum of ~122 mm mercury. Specimens were post-fixed in 1% reduced OsO_4 plus 1.5% $Fe(CN)_6$ in deionized water by microwaving for 40 s at a power setting of one and a vacuum of ~122 mm mercury. Specimens were then washed in deionized water with two 40 s pulses at a power setting of one. Specimens were dehydrated in an ascending ethanol series from 10 to 100% followed by propylene oxide (100%).

Infiltration of specimens was with ascending concentrations of Spurr's resin starting with 4% Spurr's in propylene oxide and a 3 min pulse in the microwave at a power setting of two and a vacuum of 122 mm mercury, 12% Spurr's overnight on a rotator, 25% Spurr's and a 3 min pulse in the microwave at a power setting of two and a vacuum of 122 mm mercury, 50% Spurr's 3 min microwave pulse power setting of two and 122 mm mercury, 75% Spurr's plus accelerator overnight, and 100% Spurr's for 6 h. Specimens in resin were embedded in molds and the resin polymerized for 48 h at 60 °C.

Specimens were thin sectioned using a diamond knife, mounted on formvar-coated grids and stained with 2% uranyl acetate in 70% methanol for 10 min followed by lead citrate for 3 min. Specimens were observed on a Phillips CM-10 transmission electron microscope.

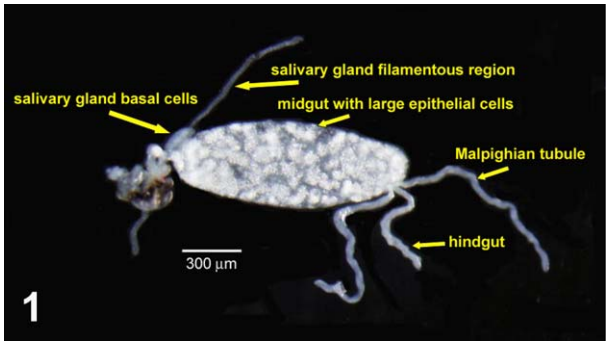

Fig. 1. Alimentary tract of a 5-day-old larva. The midgut is a simple cylindrical structure with the most conspicuous feature being its large size filled with contents and its large epithelial cells; no gastric caecae are present. In addition to the midgut a salivary gland, the single pair of Malpighian tubules, and the hindgut are visible (40×).

3. Results

3.1. Morphology and ultrastructure of the midgut

Dissection of the alimentary tract of the 1st-instar larva and analyses of the general morphology documented the Hessian fly larval midgut as a cylindrical structure composed of very large epithelial cells with the absence of gastric caecae (Fig. 1). Also visible in dissections were the salivary glands, the single pair of Malpighian tubules, and the thin hindgut. These structures are also documented in the report by Haseman (1930). Ultrastructural analysis of the midgut from a 1st-instar Hessian fly larva feeding on susceptible Newton wheat at 24 h after egg hatch confirmed the presence of microvilli protruding into the lumen; however, neither a peritrophic membrane (PM) nor a perimicrovillar membrane (PMM) have been observed to date in any larval Hessian fly midgut sections and the microvilli appear to be in direct contact with the lumen contents (Fig. 2). The microvilli also were not densely packed and had an irregular orientation. Midgut epithelial cells of 1st-instar larvae also showed extensive pools of ribosomes in the

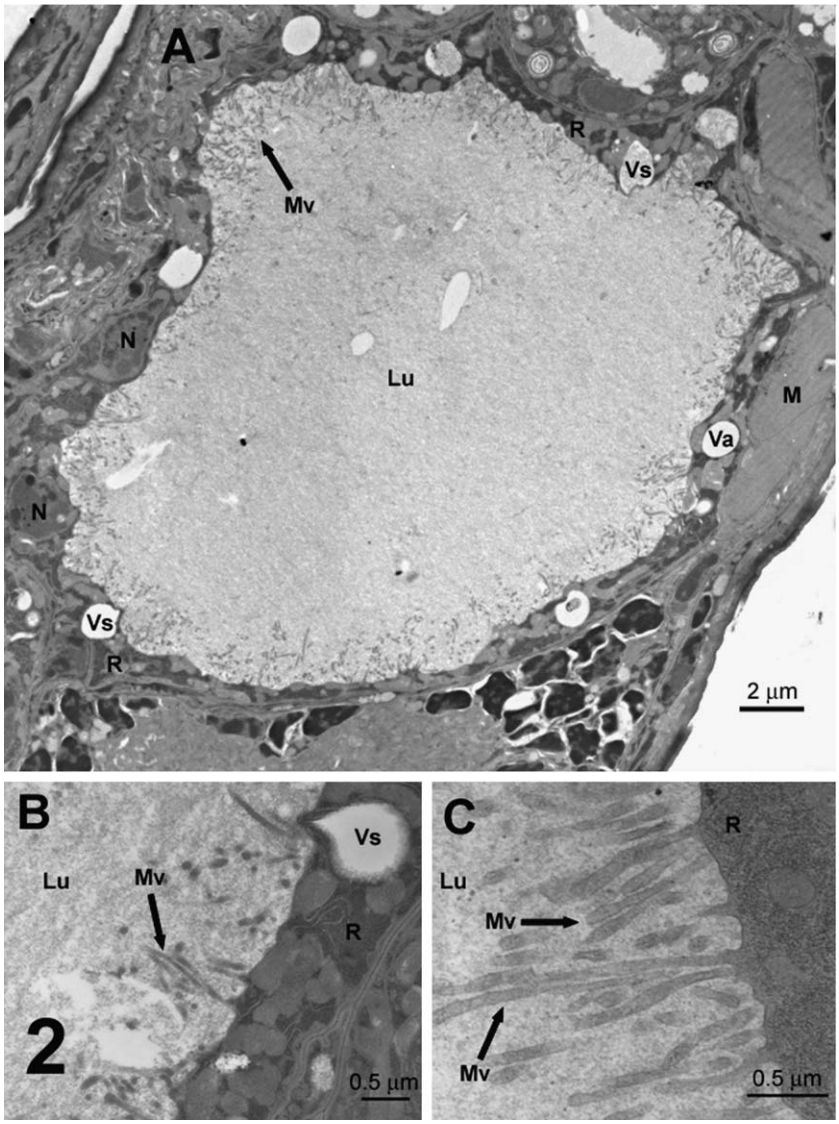

Fig. 2. Transmission electron microscopy images of the midgut of a 24-h-old larva from susceptible Newton wheat in cross section. (A) Entire midgut within the larva, arrow indicates typical microvilli, no peritrophic membrane is visible (1200×). (B) Midgut epithelial cell, arrow indicates typical microvilli projecting into the midgut lumen, a possible secretory vesicle and pools of ribosomes are also visible (20,000×). (C) Higher magnification of midgut microvilli, arrows indicates typical microvilli projecting into the lumen of the midgut, microvilli are in direct contact with lumen contents and no perimicrovillar-like membrane is visible (37,000×) Lu, lumen; Mv, microvilli; N, nucleus; Vs, vesicle; Va, vacuole; R, ribosomes; M, muscle.

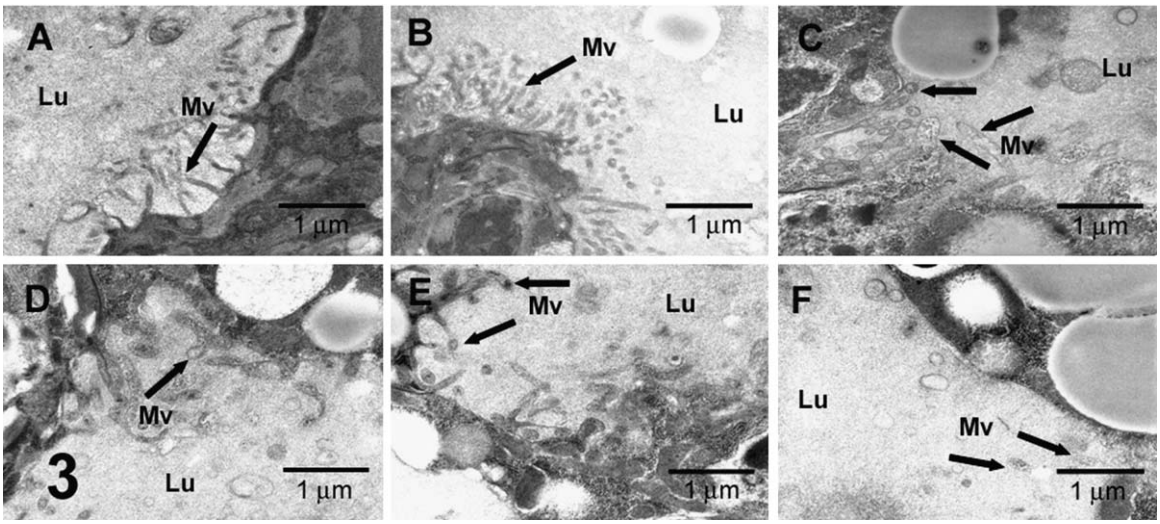

Fig. 3. Transmission electron microscopy of midgut epithelial cell microvilli from 1st-instar larvae fed on susceptible Newton wheat, subjected to starvation, or fed on resistant Iris wheat. (A) Midgut microvilli from a larva fed on susceptible Newton wheat for 3 h with normal appearing microvilli (arrow) projecting into the lumen. (B) Midgut microvilli from a larva subjected to starvation for 3 h with microvilli projecting into the lumen similar in appearance (arrow) to those present in the larva from susceptible Newton wheat. (C) Midgut microvilli from a larva that had fed on resistant Iris wheat for 3 h, arrows indicate swollen and misshapen microvilli. (D) Midgut microvilli from a second larva that had fed on resistant Iris wheat for 3 h, arrow indicates a misshapen microvillus that is possibly bifurcated and appears to have a swollen end. (E) Midgut microvilli from a third larva that had fed on resistant Iris wheat for 3 h, arrows indicate misshapen microvilli that appear to have swollen ends. (F) Midgut epithelial cell from a fourth larva that had fed on resistant Iris wheat for 3 h, epithelial cells were almost completely devoid of microvilli, arrows indicate what appear to be remnants of microvilli. All images 20,000×. Mv, microvilli; Lu, lumen.

cytoplasm as well as vacuoles and possible secretory vesicles (Fig. 2).

3.2. Ultrastructure of midguts from larvae fed on Newton, Iris, or starved

Three hours after egg hatch, midguts of larvae that had fed on susceptible Newton wheat displayed normal appearing microvilli protruding into the lumen (Fig. 3A). Midguts of larvae subjected to starvation for 3 h after egg hatch also showed microvilli protruding into the lumen contents (Fig. 3B). However, microvilli in the midguts of larvae 3 h after egg hatch that had fed on resistant Iris wheat were swollen, had bulbous termini, and were in various stages of disruption (Fig. 3C–F). Further, in some larvae the midgut was almost devoid of microvilli (Fig. 3F).

Analyses 6 h (Fig. 4A) and 24 h (Fig. 4D) after egg hatch of midguts from larvae that had fed on susceptible Newton wheat revealed a brush border of microvilli lining the midgut as previously seen in larvae from Newton (*vide supra*). Midguts of larvae that had been subjected to starvation for 6 h (Fig. 4B) and

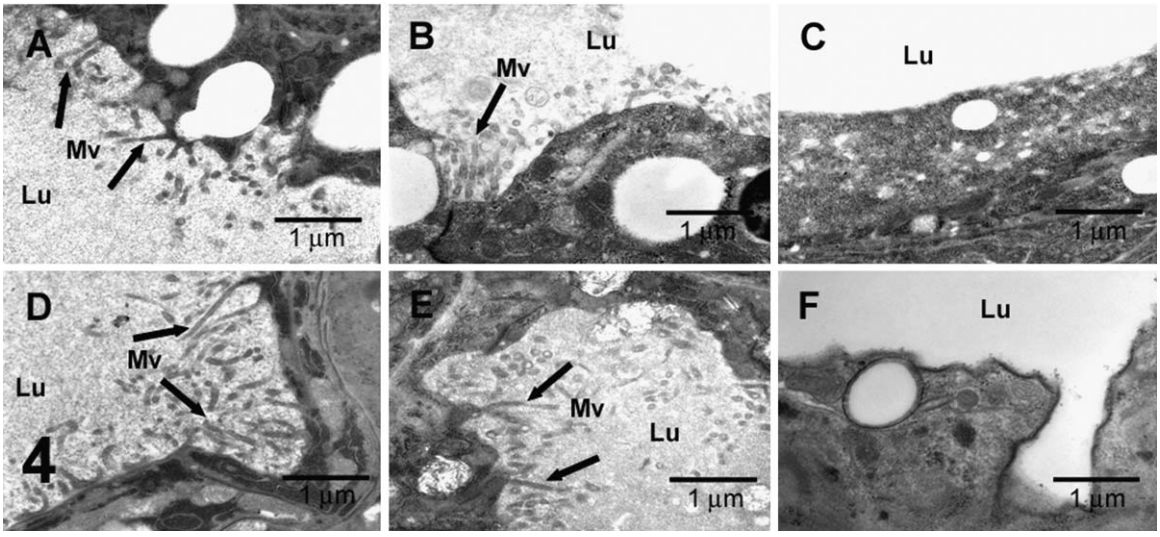

Fig. 4. Transmission electron microscopy of midgut epithelial cells and microvilli from 1st-instar larvae fed for 6- and 24-h after egg hatch on susceptible Newton wheat, subjected to starvation, or fed on resistant Iris wheat. (A) Midgut microvilli from a larva that had fed on susceptible Newton wheat for 6 h with normal appearing microvilli (arrows) projecting into the lumen. (B) Midgut microvilli from a larva subjected to starvation for 6 h with microvilli projecting into the lumen similar in appearance (arrow) to those present in the larva from susceptible Newton wheat. (C) Midgut epithelial cell of a larva that had fed on resistant Iris wheat for 6 h, no microvilli are present and the lumen appears devoid of contents. (D) Midgut microvilli from a larva that had fed on susceptible Newton wheat for 24 h with normal appearing microvilli (arrows) projecting into the lumen. (E) Midgut microvilli from a larva subjected to starvation for 24 h with microvilli projecting into the lumen similar in appearance (arrows) to those present in the larva from susceptible Newton wheat. (F) Midgut epithelial cell from a larva that had fed for 24 h on resistant Iris wheat, no microvilli are present and the lumen appears devoid of contents. All images 20,000×. Mv, microvilli; Lu, lumen.

24 h (Fig. 4E) after egg hatch also had normal appearing microvilli forming the brush border, and surprisingly the lumens were not empty. However, 6 h (Fig. 4C) and 24 h (Fig. 4F) after egg hatch midguts of larvae that had fed on resistant Iris wheat were devoid of microvilli and lacked lumen contents.

4. Discussion

The present study documents the midgut of the larval Hessian fly as a simple cylindrical structure unlike the tubular sinusoidal structure found in *D. melanogaster* and other dipterans. Additionally, the microvilli forming the brush border or striated border of the midgut did not appear to be as numerous or to have the parallel orientation found in *D. melanogaster* and other insect species. Further, a PM was absent from the midgut and the microvilli appeared to be in direct contact with lumen contents.

The midgut of the larval Hessian fly appears to be unique among the dipterans in that a PM is absent, while to the best our knowledge the other dipterans examined to date all have a well developed PM. True PMs are reported as absent in the hemipterans that subsist solely on plant saps; however, hemipteran insects do have an extra-cellular lipoprotein membrane, the PMM, ensheathing the midgut microvilli (Terra, 1988). Modified PMMs are found in the Aphididae at the apices of the microvilli as amorphous membrane masses (Cristofoletti et al., 2003; Silva et al., 2004) and membranes similar to PMMs have been shown to be present in the order Thysanoptera (Del Bene et al., 1991; Silva et al., 2004). However, PMMs are apparently absent in the orders Psocoptera and Phthiraptera (Silva et al., 2004).

In addition to lacking a PM, no other protective barrier, such as the PMM found in the hemipterans and thysanopterans, was present in the Hessian fly larval midgut. The need for such protective barriers in the midgut could be predicated by the mechanism of feeding employed by larvae. While Hessian fly larvae feed on cell sap from the host plant as do the hemipterans and thysanopterans unlike these insects Hessian larvae rapidly, within 24 h, alter the permeability of host-plant cells (Shukle et al., 1992; Subramanyam et al., 2007) and reprogram host-plant tissues to generate a nutritive tissue layer to provide nutrient enhanced cell sap for the developing larvae to feed upon (Harris et al., 2006). The Cecidomyiidae represents the sixth largest family of dipterans and it should be of interest to know whether the lack of a PM or a PMM protecting the microvilli in the midgut is common within the Cecidomyiidae or unique to the Hessian fly.

Among the compounds that play a role in plant defense against herbivores and pathogens are lectins (Murdock and Shade, 2002). Sites where dietary lectins could elicit antifeedants and insecticidal activities include: (1) sensory receptors, where lectins could bind to or disrupt the integrity of sensory membranes and interfere with food detection (Murdock and Shade, 2002); (2) the PM where lectin binding has been shown to cause disruption and formation of large holes (Harper et al., 1998; Hopkins and Harper, 2001); and (3) the midgut epithelial cell microvilli forming the brush border where ultrastructural studies have shown disruption of the microvilli brush border region (Harper et al., 1998; Powell et al., 1998; Li et al., 2009). The absence of an observed PM in the midgut of the larval Hessian fly should indicate the microvilli brush border as a major target for toxic lectins in the defense response of resistant wheat to larval Hessian fly attack.

The vulnerability and the rapidity of disruption, within 3 h, of the microvilli brush border of larvae feeding on resistant Iris wheat was surprising. Further, no microvilli were observed in the presumptive midguts of larvae that had fed on Iris wheat for 6 and 24 h. Since microvilli are a diagnostic feature of the midgut we presumed the large cavity observed in cross sections of larvae from resistant Iris wheat was the midgut devoid of microvilli. These results suggested the midgut microvilli brush border as a major target for toxic compounds elicited in the defense response of Iris wheat. Additionally, the presence of normal appearing microvilli in the midguts of larvae subjected to starvation further supported the disruption of the midgut microvilli brush border was due to toxic compounds in the plant's defense response and not to starvation.

Hessian fly larvae do initially imbibe limited amounts of cell sap on resistant wheat (Shukle et al., 1990) and the defense response to larval Hessian fly attack in Iris carrying *H9* involves the rapid up-regulation of two genes encoding lectins; *Hessian fly responsive gene 1* (*Hfr-1*) (Subramanyam et al., 2006) that encodes a jacalin-like lectin and *Hessian fly responsive gene 3* (*Hfr-3*) that encodes a lectin-like protein showing 68–70% identity to WGA (Giovanini et al., 2007). Due to the obligate nature of Hessian fly larvae as parasites of wheat and related grasses a viable bioassay has not been developed to directly ascertain the effects of toxic compounds on larvae. However, by Western analyses Hessian fly larvae have been shown to imbibe the HFR1 protein from wheat plants and the protein HFR1 has been shown to have antifeedant and insecticidal activities when fed to *D. melanogaster* larvae with the concentration at which 50% of the larvae died (LD_{50}) calculated to be 6.55 ± 0.15 $\mu g\ g^{-1}$ diet (Subramanyam et al., 2008). Studies to detect the presence and locations of HFR1 protein in the midguts of larvae by immunogold detection have been complicated by background with pre-immune serum and are beyond the scope of the present study. To date, there are no data as to the effects of HFR1 on midgut microvilli in *D. melanogaster*. However, given the insecticidal activity of HFR1 and the effects of WGA on midgut microvilli in *D. melanogaster* and snowdrop lectin on the midgut microvilli of the rice brown planthopper it seems plausible that both HFR1 and HFR3 proteins should have pivotal roles in the defense response of Iris wheat and one or both could be associated with the disruption of microvilli documented in the present study.

The lack of observable lumen contents in the midguts of larvae that had fed on Iris wheat for 6 and 24 h was also surprising considering the apparent presence of lumen contents in larvae subjected to starvation for 6 and 24 h. Initially this was thought to be an anomaly associated with loss of lumen contents during piercing of the anterior end of larvae to allow penetration of fixative to the midgut. However, these data were consistent as was the presence of lumen content in larvae from Newton wheat and larvae subjected to starvation suggesting that a toxic compound or compounds, such as the HFR1/HFR3 proteins, associated with the plant's defense response could cause larvae to purge their gut. The apparent lack of lumen contents in the midguts of larvae on resistant Iris wheat was also supported by the previous report of failure to recover bacteria from midguts of larvae that had fed on Iris wheat, while a diversity of bacteria where recovered from the midguts of 1st- and 2nd-instar larvae that had fed on susceptible Newton wheat (Mittapalli et al., 2006).

While it was initially thought production of H_2O_2 and other reactive oxygen species were not associated with the defense response in resistant Iris wheat to larval Hessian fly attack (Giovanini et al., 2006) analysis of the transcriptome of the larval Hessian fly by Mittapalli et al. (2007a) indicated larvae feeding on resistant Iris wheat were under oxidative stress. Further, recent data has revealed that H_2O_2 does accumulate at the site of larval attack in Iris wheat and the transcript levels of class III peroxidases rapidly increase (Liu et al., 2009). Thus, the purging of midgut lumen contents and the disruption of midgut flora also could be associated with the presence of reactive oxygen species generated during the plant's defense response.

The mode of resistance in wheat to larval Hessian fly attack is antibiosis; however, while not feeding or developing normally avirulent larvae infesting Iris wheat do survive for 5–6 days. This has been interpreted as indicating that resistant wheat is in effect

starving the larvae to death. The presence of microvilli at the luminal surface of midgut epithelial cells greatly increases the surface area for secretion of enzymes and absorption of nutrients. Thus, the disruption of the microvilli brush border in larvae on Iris wheat, while perhaps not causing rapid mortality, should result in a major physiological loss of function in digestion and absorption of nutrients by 1st-instar larvae. This in turn could contribute toward the lack of development of avirulent larvae on Iris wheat and their ultimate demise.

The goal of the present study was to discover if changes occurred in the ultrastructure of midgut epithelial cells in Hessian fly larvae feeding on resistant Iris wheat compared to larvae feeding on susceptible Newton wheat and to larvae subjected to starvation. Results documented the midgut microvilli brush border as a major site of action for toxic compounds in the defense response of Iris wheat to larval Hessian fly attack. Among the possible toxins involved in disruption of the microvilli brush border in larvae feeding on Iris wheat are the lectin protein HFR1 and the lectin-like protein HFR3. Additionally, the production of reactive oxygen species such as H_2O_2 during the plant's defense response could also be involved in disruption of microvilli and the apparent loss of lumen contents. While the midgut has been documented as a major site of action for toxins in the defense response of Iris wheat to Hessian fly attack and disruption of the microvilli brush border as one of the mechanisms resulting in antibiosis other sites such as the sensory membranes of gustatory receptors could also be targets of lectins and other toxic plant compounds. The lack of a protective PM in the midgut of larvae also could imply microvilli are particularly vulnerable to plant toxins and subject to rapid disruption. These results provide the first documentation of the midgut as a major site of action for toxic plant compounds in the wheat/Hessian fly interaction and advance our knowledge of the basis of antibiosis in this interaction.

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